
Citation:

Pollock, NL and Rai, M and Simon, KS and Hesketh, SJ and Teo, ACK and Parmar, M and Sridhar, P and Collins, R and Lee, SC and Stroud, ZN and Bakker, SE and Muench, SP and Barton, CH and Hurlbut, G and Roper, DI and Smith, CJI and Knowles, TJ and Spickett, CM and East, JM and Postis, V and Dafforn, TR (2019) SMA-PAGE: A new method to examine complexes of membrane proteins using SMALP nano-encapsulation and native gel electrophoresis. *Biochim Biophys Acta Biomembr*, 1861 (8). pp. 1437-1445. ISSN 1879-2642 DOI: <https://doi.org/10.1016/j.bbamem.2019.05.011>

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SMA-PAGE: A new method to examine complexes of membrane proteins using SMALP nano-encapsulation and native gel electrophoresis

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Abstract

Most membrane proteins function through interactions with other proteins in the phospholipid bilayer, the cytosol or the extracellular milieu. Understanding the molecular basis of these interactions is key to understanding membrane protein function and dysfunction. Here we demonstrate for the first time how a nano-encapsulation method based on styrene maleic acid lipid particles (SMALPs) can be used in combination with native gel electrophoresis to separate membrane protein complexes in their native state. Using four model proteins, we show that this separation method provides an excellent measure of protein quaternary structure, and that the lipid environment surrounding the protein(s) can be probed using mass spectrometry. We also show that the method is complementary to immunoblotting. Finally we show that intact membrane protein-SMALPs extracted from a band on a gel could be visualised using electron microscopy (EM). Taken together these results provide a novel and elegant method for investigating membrane protein complexes in a native state.

Key words

Membrane protein
Nanoparticle
SMALP
Native PAGE
Protein complex

Abbreviations

ABC	ATP-binding cassette
AcrB	acriflavine resistance protein B
AUC	analytical ultracentrifugation
Bam	β -barrel assembly machinery
BSA	bovine serum albumin
EM	electron microscopy
PAGE	polyacrylamide gel electrophoresis
Sav1866	<i>Staphylococcus aureus</i> protein 1866
SDS	sodium dodecylsulphate
SEC	size exclusion chromatography
SMA	styrene maleic acid
SMALP	styrene maleic acid lipid particle

Introduction

Defining their quaternary structure is fundamental to understanding the function of many proteins. One key tool used for this is native polyacrylamide gel electrophoresis (native PAGE)^{1,2}. This method has been successful for a wide range of applications where understanding protein quaternary structure has implications for both health³ and disease^{4,5}. In particular, native PAGE provides a non-disruptive and elegant method for defining the quaternary structure of small samples of protein.

For membrane proteins native PAGE has typically been less reliable. One issue is the necessity of using detergent during the protein solubilization and PAGE processes: the detergent interferes with non-covalent interactions between protein subunits⁶, potentially disrupting quaternary structures. While conventional native PAGE in the presence of detergent has yielded formative data, for instance in defining the subunit composition of mitochondrial complexes⁷, in many cases native PAGE gels of membrane proteins have low clarity and resolution, rendering them difficult to interpret. To mitigate these issues, current native PAGE protocols for membrane proteins typically require other excipients alongside the detergent (e.g. Coomassie dye for blue native PAGE, or proprietary additives)⁸, which at best make the method complex and unwieldy and at worst fail to improve the quality of the data. To more reliably and rapidly expand our understanding of membrane protein complexes, we need a simple and effective native PAGE system that preserves the native state oligomeric state of membrane proteins.

In 2009 we developed a new approach to membrane protein extraction that replaced conventional detergents with an amphipathic polymer, styrene maleic acid (SMA)⁹. SMA can extract proteins directly from the membrane complete with their local lipid environment¹⁰. The resulting SMA lipid particles (SMALPs) contain membrane proteins in stable nanoparticles of ~10 nm diameter, which are suitable for many downstream biochemical studies. The SMALP method has been successful for ion channels¹¹, transporters^{12,13}, enzymes¹⁴, respiratory complexes¹⁰ and light harvesting¹³ complexes, and receptors¹⁵. These proteins have been extracted from a range of biological membranes. In all of these cases the SMALP, unlike detergent, leaves the quaternary structure of the protein intact. The SMA molecule is amphipathic, and its maleic acid moieties have two logarithmic acid dissociation constants (pKa) at pH 1.9 and 6.1. Therefore at the pH used for Tris-glycine PAGE, SMA is negatively charged, making it analogous to the sodium dodecyl sulphate in standard denaturing gels. Crucially, SMA differs from SDS in that it does not bind to and unfold the protein. Instead it interacts only with the lipid bilayers that surround the membrane protein, maintaining the membrane and hence

stabilising the membrane protein structure ^{12,13}. This makes SMA highly unlikely to interrupt protein-protein contacts that stabilise membrane protein complexes. Indeed, studies using SMALPs have shown that it preserves protein complexes including homo-oligomers ^{11,16} and hetero-complexes, e.g. cytochromes ¹⁰, light harvesting complexes¹³, and protein translocons ^{17,18}. More importantly for electrophoresis, the high negative charge density of the SMA polymer in the SMALPs should enable membrane proteins, regardless of their inherent charge at pH 8, to migrate under electrophoresis. It is also worth noting that SMALPs are typically of uniform size, regardless of the mass of protein in the particle. This means that differences in migration observed in electrophoresis are likely to be dominated by the size of the protein and not the charge on the particle.

In this work we have developed a native PAGE method for use with SMALPs (SMA-PAGE). To do so, we chose a set of membrane proteins with different architectures and oligomeric states that can be routinely purified in SMALPs (Supplementary Table 1). These are: ZipA, a 36 kDa single transmembrane helix protein that adopts a range of oligomeric forms including monomers ¹⁹; Sav1866, a 65 kDa ABC-transporter that functions as a homodimer ²⁰; the 112 kDa bacterial drug transporter AcrB that assembles as a homotrimer ²¹; and the microbial protein translocator complex Bam, a hetero-pentamer with a stoichiometry of 1:1:1:1:1 and a total mass of ~211 kDa ²². We assessed whether these proteins migrated as intact SMALPs, whether their migration was consistent with their native oligomeric states, and whether they retained lipids. We also showed that protein stains (e.g. Coomassie) and immunostains could be used with the system. We also proved that intact SMALPs could be extracted from the gel and be used for proteomics and lipidomics analysis. Finally we showed that the intact particles extracted from the SMA-PAGE could be visualised using electron microscopy.

Results

The aims of this study were three-fold. First, we aimed to discover whether SMALP particles migrated intact through a native electrophoresis system, and whether their migration was related to the mass of the protein encapsulated in the SMALP. Secondly, we asked whether proteins in the SMA-PAGE gels could be easily visualised. Finally we explored the compatibility of SMA-PAGE with downstream analysis of membrane proteins.

Proteins in SMALPs migrate under native electrophoresis

The SMALP-encapsulated membrane proteins selected for this study were applied to a 4-20 % acrylamide tris/glycine gel in a non-denaturing, non-reducing load dye. Their migration through the gel was compared to native electrophoresis standard proteins (Figure 1) and the experimentally calculated molecular weights are shown in the inset table. Each protein migrated to a position consistent with its molecular weight and produced a distinct band when stained with Coomassie-like protein stain.

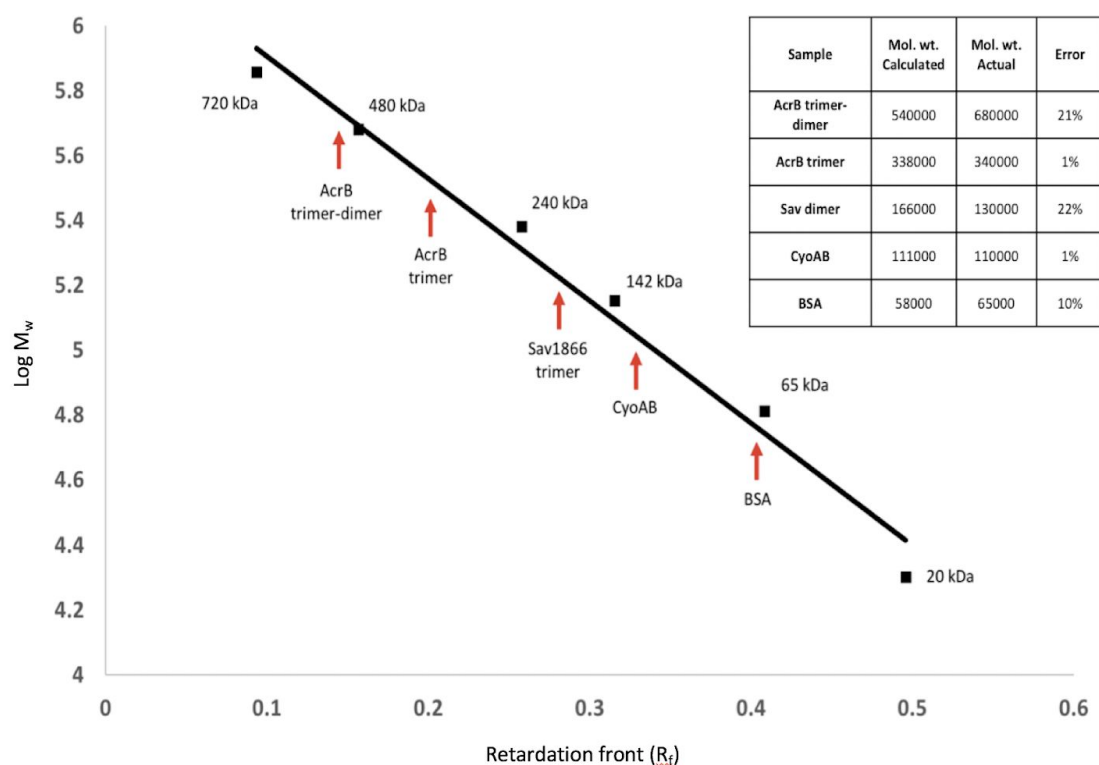


Figure 1: Retardation front (Rf) analysis of an SMA-PAGE native gel. The migration (Rf) of the native protein markers was plotted against their molecular weights (black squares and labels) and fitted with a straight line. The Rf value of each protein in SMALPs was plotted on this line (red star) and its molecular weight calculated using the equation of the best fit line. The experimentally calculated molecular weights are shown in the inset table alongside their actual molecular weights

The migration of the model proteins was compared on SDS-PAGE and SMA-PAGE gels (Figure 2). Qualitatively, protein bands on SMA-PAGE (native) gels were less intensely stained than bands on SDS-PAGE gels loaded with equivalent amounts of protein (Figure 2). This implied that less of the protein surface area was available for staining in the SMA-PAGE gels compared to denaturing gels, which agrees with the fact that the majority of the protein remained

embedded in a lipid bilayer in the particle. The bacterial drug efflux transporter, AcrB, assembles as a trimer of three 113 kDa subunits. In SDS-PAGE, there was a single band consistent with the subunit molecular weight (Figure 2A). In SMA-PAGE, the migration of AcrB confirmed that it was present as a trimeric assembly of ~340 kDa (Figure 2A). Higher molecular weight bands were also visible at ~700 kDa. The higher molecular weight band seen in the SMA-PAGE experiment agreed our own previous observation that trimer-dimers of AcrB exist in SMALPs as observed by negatively-stained EM ¹⁶. It is likely that the dimer is mediated by intraparticle protein-protein interactions. The Bam ABCDE complex purified in SMALPs was also analysed. SDS-PAGE showed that the complex consisted of the outer membrane beta-barrel Bam A (90.5 kDa) and its associated soluble lipoproteins BamB-E of 42, 37, 28, 12 kDa respectively (Figure 2B). In SMA-PAGE the Bam complex migrated principally at ~240 kDa which probably represented the 1:1:1:1:1 stoichiometry of its subunits (Figure 2B). However, two additional bands were observed in this gel at 600 and 140 kDa. A previous report of Bam complex purified in detergent and resolved by blue native PAGE (BN-PAGE) showed that there were several populations of Bam complexes, composed of differing subunits that migrated to 500, 300 and 150 kDa depending on the detergent concentration of the sample²³. As we have shown, SMA-PAGE supported this observation.

In the case of the *Staphylococcus aureus* ABC protein Sav1866, the protein-SMALP assembly migrated on SMA-PAGE gels as a physiological homodimer of 10 kDa (Figure 2B), compared with 65 kDa on SDS-PAGE (Figure 2C). By contrast, the bacterial cell division protein ZipA migrated in SMA-PAGE as a number of different oligomeric species between approximately 60 and 720 kDa. This reiterates previous native PAGE analysis of full-length ZipA solubilised using detergent and the truncated soluble region of ZipA, showing the presence of a number of different oligomers ²⁴.

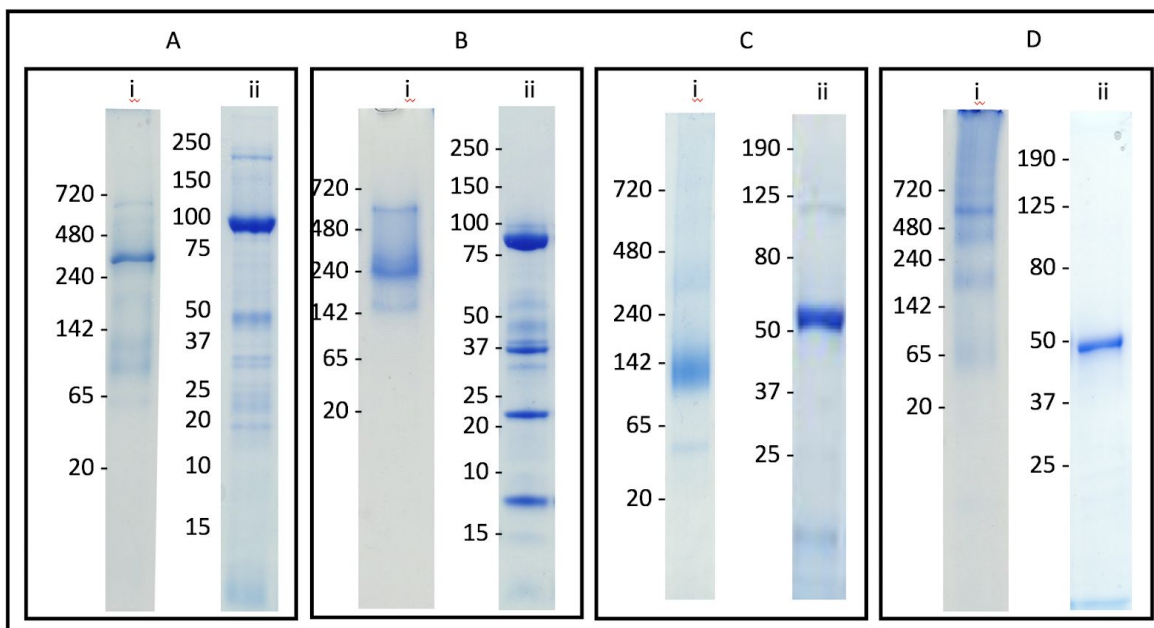


Figure 2: PAGE gels of membrane proteins with different architectures and molecular weights. AcrB (A), Bam complex (B) Sav1866 (C) and ZipA (D) analysed by SMA-PAGE (i) and SDS-PAGE (ii). The positions of molecular weight markers (in kDa) are indicated. The full-sized gels are shown in Supplementary Figure 1.

To further confirm that the band observed on SMA-PAGE contained all the components of the Bam complex, the band at 220 kDa was excised from the SMA-PAGE, incubated with denaturing load dye, heated to 85 °C for 10 minutes and subjected to SDS-PAGE. After Coomassie staining each of the Bam subunits were visible (Supplementary Figure 2). To further confirm its identity, part of the excised band was submitted for protein identification by mass spectrometry (Supplementary Table 3). These results confirmed that the Bam complex migrated under SMA-PAGE in its fully assembled form, and its lipoprotein subunits maintained contact with the BamA-SMALP throughout SMA-PAGE. In addition, mass spectrometry revealed that the folding chaperone protein SurA was also present in the Bam complex sample (Supplementary Table 2). With a molecular weight of 47 kDa, the addition of this protein to the core Bam complex assembly could account for the higher-than-expected molecular weight observed from the native gel (Figure 2Bi).

Protein-SMALPs retain their lipid environment during SMA-PAGE

Protein-SMALPs can be separated by native PAGE, but it was important to determine whether the nanoparticles retained lipid as well as protein in the polyacrylamide gel. In this section we

demonstrate that protein and lipid co-migrated as intact SMALPs. Lipids were recovered from gels and could be identified by mass spectrometry.

Bands corresponding to the protein-SMALPs were excised from SMA-PAGE gels. The resulting samples, which contained intact protein-SMALP, were extracted into chloroform/methanol. Liquid chromatography-mass spectrometric analysis of these extracts demonstrated that lipids were retained with the protein-SMALPs after migration through the native gels (Figure 3). A variety of molecular species belonging to phosphatidylethanolamine and phosphatidylglycerol classes (i.e two major membrane lipid classes of *E. coli*) were detected (Figure 3). The phospholipid profile was very similar to that of a global extract of *E. coli* (Teo et al, 2019).

By contrast, in extracts from identical samples resolved by SDS-PAGE, no lipid was detected. This result had two clear implications. Firstly, it demonstrated that the SMALPs migrated in the SMA-PAGE in an intact form retaining the protein, lipid and SMA polymer, which is an entirely novel observation. Secondly, the results showed that mass spectrometry can be used on microgram quantities of sample extracted from SMA-PAGE gels to provide not only the identity of the protein, but also information about the lipid environment in which it is found.

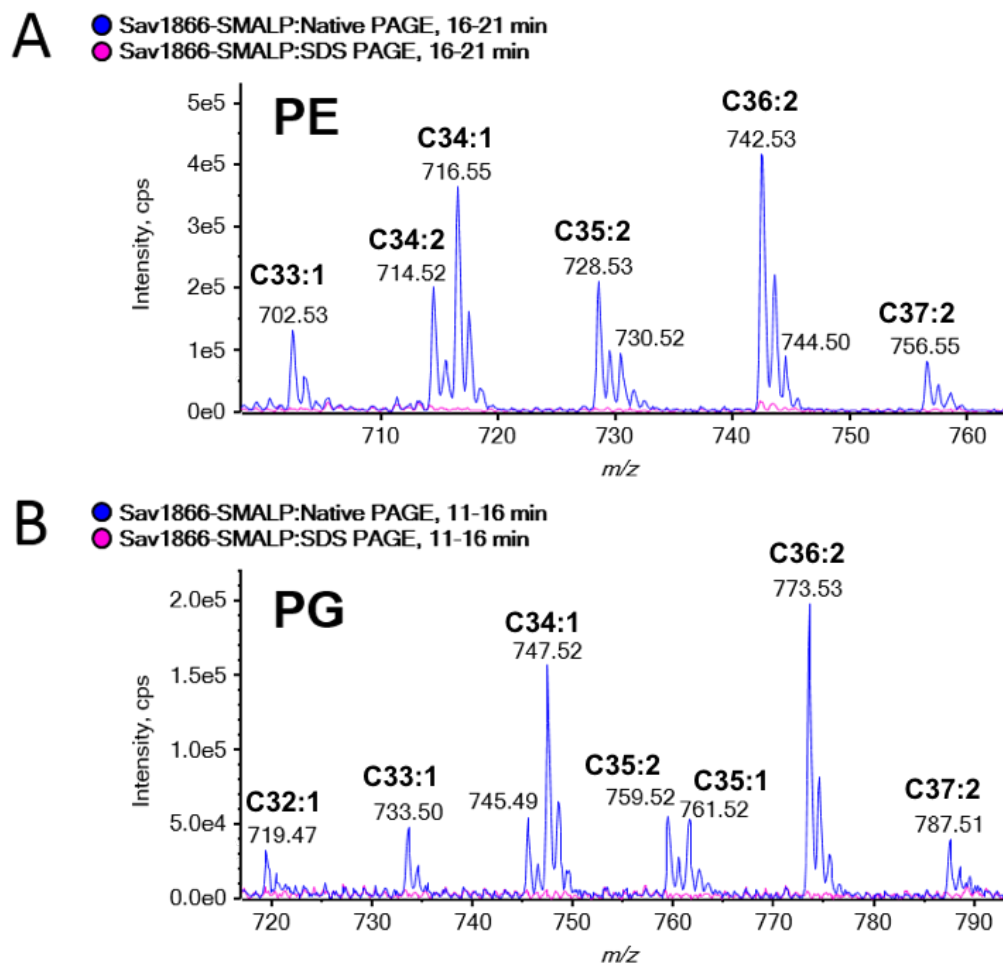


Figure 3: Evidence for lipids co-migrating with protein-SMALPs. Proteins bands resolved by SDS-PAGE (pink) and SMA-PAGE (blue) were extracted into SDS. Lipids were extracted using a modified Folch method and characterised by liquid chromatography coupled to mass spectrometry (LC-MS) in negative ion mode. The phospholipids were identified by their mass-to-charge ratios (m/z) and retention times on reverse phase chromatography. The spectra correspond to the region 16-21 mins of the chromatogram where phosphatidylethanolamines eluted (A), and the region from 11-16 mins where phosphatidylglycerols eluted (B). The individual signals are annotated with the total number of carbons in the fatty acyl chains (XX) to number of double bonds (Y) according to the formula CXX:Y.

SMA-PAGE can be used to for quality control of membrane protein samples

A number of downstream analysis techniques commonly applied to studies of proteins rely on the production of a mono-dispersed sample, for example structural biology techniques like X-ray

crystallography, electron microscopy and small-angle scattering. Many methods that assess sample heterogeneity use size-based separation (e.g. size exclusion chromatography, analytical ultracentrifugation) which require significant amounts of concentrated protein. The separation of protein SMALPs in SMA-PAGE offers an opportunity to rapidly assess quality of membrane protein sample using small amounts of sample.

To assess the potential for SMA-PAGE for quality control of protein samples, samples containing Sav1866-SMALPs were analysed using several methods. First, Sav1866-SMALPs were separated by size exclusion chromatography (SEC, Supplementary Figure 3). These fractions were resolved on SDS-PAGE to show a dominant band at 65 kDa in each fraction (Figure 4). By contrast, the same fractions analysed by SMA-PAGE gel showed multiple bands, ranging from protein that did not exit the loading wells down to bands at ~130 kDa (Sav1866 dimers). This corresponded with the SEC profile of the samples, which indicated that the sample injected onto the column had contained aggregated Sav1866-SMALPs along with multiple oligomers. It was also clear that SMA-PAGE outperformed SEC in resolving protein-SMALPs, since all the fractions contained oligomers that were further separated by SMA-PAGE. Finally, several fractions from the SEC separation were analysed by sedimentation velocity analytical ultracentrifugation (svAUC, Supplementary Figure 4). This corroborated the observation that fractions across the SEC profile contained protein of different molecular weights despite it consisting of highly pure Sav1866 (Figure 4, Supplementary Figure 4). Overall, the SMA-PAGE provided a fast and convenient method by which to assess the sample monodispersity.

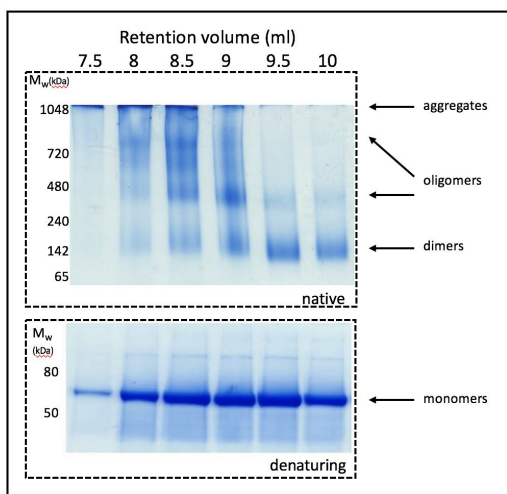


Figure 4: Fractions containing Sav1866 after size exclusion chromatography analysed by SMA-PAGE (upper) and SDS-PAGE (lower). Fractions eluted from a Superdex200 10/300 column were run on both native and denaturing gels.

Immunoblotting is compatible with SMA-PAGE

The applications of SMA-PAGE demonstrated in the preceding sections used samples of pure protein, but in many cases it is preferable to assess the oligomerization state of proteins in impure samples (e.g. crude cell extracts). With some small adjustments, immunoblotting was used to identify proteins in different oligomeric forms in crude SMA-solubilised fractions.

Solubilised membranes were separated by SMA-PAGE and transferred to a nitrocellulose membrane for immunoblotting. In the case of ZipA, this revealed that the high molecular weight species observed by SMA-PAGE were oligomeric ZipA-SMALPs (Figure 5). ZipA could be identified in both purified fractions and in a crude solubilised membrane fraction. The transfer efficiency of the blot was enhanced by soaking the gel in standard SDS running buffer for 10 minutes before transfer to the membrane (Supplementary Figure 5). Samples containing high concentrations of SMA (i.e. solubilised membranes) initially showed high nonspecific antibody binding (Supplementary Figure 5), which was reduced by addition of 25 mM L-arginine to the blocking buffer or by undertaking a buffer exchange in a spin concentrator of the crude solubilised membranes prior to running the SMA-PAGE. To achieve good signal, it was also necessary to incubate antibody with the nitrocellulose membrane for 48 hours. These results showed that with relatively minor modifications to the existing method, immunoblotting techniques were compatible with SMA-PAGE.

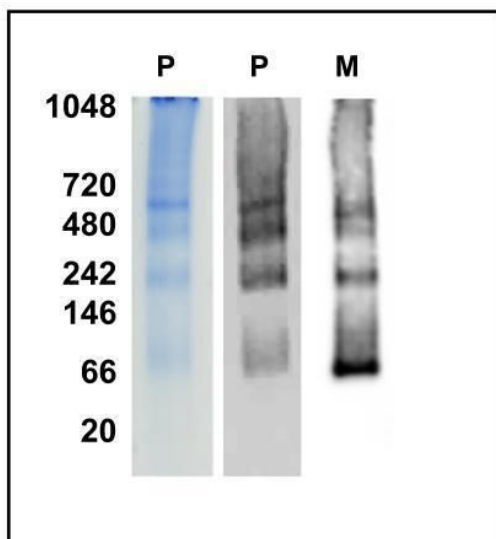


Figure 5: Detection of ZipA-SMALPs by immunoblotting. Purified ZipA-SMALPs (P) resolved by native PAGE were detected by Coomassie staining (blue) and immunoblotting (grey). The crude SMA-solubilised membrane fraction (M) was also resolved by native PAGE and ZipA was detected by immunoblotting against its His tag.

SMA-PAGE is a rapid and efficient method of sample preparation for electron microscopy

Cryo-electron microscopy (cryoEM) is now capable of delivering atomic-resolution data on protein structures and in recent years many protein structures, including those of membrane proteins, have been solved using this method. We have shown that SMALP-proteins are amenable to cryoEM^{16,25,26} and recently the first atomic resolution structure of a SMALP protein has been resolved²⁷. One particularly attractive feature of cryo-EM is its low demand for sample quantity; grids can be prepared using microgram amounts of protein. Nonetheless it still requires monodisperse protein samples. Here we show how SMA-PAGE can be used to produce such samples, which offers the potential to access structural data for hard-to-produce proteins,

Samples of AcrB and Sav1866 were prepared for electron microscopy (EM) by running two samples of each on SMA-PAGE in neighbouring lanes. For each protein, one lane of the gel was stained using Coomassie stain to establish the position of the band, while the other was not stained. In one experiment the corresponding region in the unstained lane was excised and the protein extracted into a simple buffer of 20 mM Tris-Cl pH 8 and 150 mM NaCl. This solution, containing the SMALP-solubilised membrane protein, was used to prepare negatively-stained grids by conventional methods¹⁶ and imaged by EM (Figure 6A). This shows that intact particles of monodisperse protein can be recovered from SMA-PAGE gels. Alternatively, AcrB was successfully transferred directly from the gels onto glow-discharged carbon/formvar coated copper grids for negative staining and imaging (Figure 6B). In the latter case, the band used for the preparation of EM grids was analysed by mass spectrometry to confirm the identity of the protein(s) it contained (Supplementary Table 4).

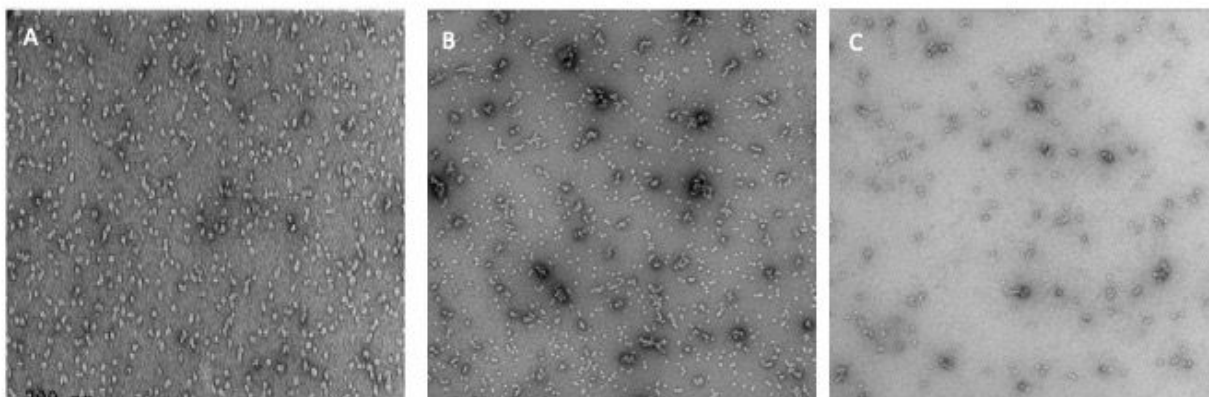


Figure 6: Micrographs of negatively stained Sav1866 (A) and AcrB (B,C). Each protein sample underwent SMA-PAGE and was extracted into buffer (Sav1866, A) or directly blotted from the surface of the gel onto a glow-discharged grid (AcrB, B).

Discussion

Determining the quaternary state of membrane proteins is a continuing challenge for protein biochemists. Without this information our understanding of the dynamics of protein complexes in membranes is incomplete. This in turn leaves a large gap in our understanding of processes as important as endocytosis and cell signalling. Central to this issue has been the use of detergents, which have frequently been shown to alter the oligomeric state of membrane proteins making the results from conventional hydrodynamic techniques untrustworthy. Similarly methods based on cross-linking of proteins *in situ* in membranes are dogged by artifacts. The SMALP system fundamentally resolves these issues by extracting particles without directly interacting with the protein complex. In this study we have exploited the mild solubilisation method offered by SMA and combined it with native gel electrophoresis to generate a new method for studying membrane protein assembly.

We have applied our SMA-PAGE technique to four well-characterised membrane proteins with diverse architectures to demonstrate that proteins in SMALPs migrate consistently under native gel electrophoresis (SMA-PAGE). The migration is due to the high negative charge density of the maleic acid groups in the SMA copolymer at the pH used for the electrophoresis. The migration of the protein-SMALPs shown here correlates exceptionally well with their known molecular weights and oligomeric states. We anticipated that migration through the gel would also be dependent on the hydrodynamic properties of the protein and there may also be a mass contribution from the SMA and associated lipids. In the case of the elongated ABC protein Sav1866, migration was indeed slower than globular BSA despite the similar sizes of their dimeric states. However, in general, the molecular-weight dependence of migration in SMA-PAGE appears to be similar to that of SDS-PAGE.

In SDS-PAGE, SMA migrates separately from the protein and is sensitive to standard protein stains²⁸. Consequently it is observed on gels at a molecular weight of around 5-10 kDa. This staining was not observed in the native gels. This indicated that SMA remained associated with the protein during electrophoresis. Likewise, we have shown that membrane lipid can be extracted from protein bands after SMA-PAGE, but not after SDS-PAGE of the same samples. Therefore we conclude that the SMALPs migrate as a discrete complex containing protein, lipids and SMA. Due to the small size of the SMA polymer relative to the sizes of the proteins under study, and the accuracy of the mass estimates derived from SMA-PAGE (Figure 1), no mass contribution to the overall assemblies could be directly attributed to the polymer.

At present our understanding of the influence of native lipids on membrane protein structure and function is at a nascent stage. The combination of SMALPs, SMA-PAGE and mass spectrometry provides a powerful new tool to explore this further using small amounts of protein. Mass spectrometry of protein SMALPs has already revealed differences in the lipid preferences of bacterial cell division proteins [Teo et al 2019]; integrating the SMA-PAGE improves the purity of samples under analysis and may reveal further information on protein-lipid interactions. In addition to this, proteins extracted from SMA-PAGE gels and imaged by EM display the characteristic disc-like appearance of the samples prior to electrophoresis.

The initial observation that SMALPs promote the migration of membrane proteins under native conditions has many implications. Using SMA-PAGE, we resolved protein-SMALPs of different sizes, leaving oligomers (AcrB, Sav1866) and complexes (BamABCDE) intact. The intact migration of the Bam complex, consisting of both membrane-spanning domains and lipoprotein subunits, revealed the power of this method to advance our understanding of large membrane protein complexes. This may also allow us to examine, in a physiologically-relevant manner, the integration of membrane proteins into other cellular processes via soluble binding partners. On a more prosaic level, **this method also allows purification contaminants to be distinguished from binding partners, a distinction that cannot be made when proteins are resolved on denaturing gels. Indeed, since proteins can be extracted from gels after SMA-PAGE, this may be useful as an additional purification step: bands containing the protein of interest can be excised and extracted into native buffers for downstream analysis.**

In addition, SMA-PAGE gels of fractions from an SEC column clearly showed differential migration of pure but polydisperse samples. The migration of protein on the gels corresponded well with the retention volumes from SEC columns. Aggregated material failed to migrate into the gel at all. This provides a fast and efficient quality control step for protein samples prior to applications such as EM, crystallisation and scattering techniques, especially after protein samples have been stored or freeze/thawed. Similarly, the results from SMA-PAGE for Sav1866, AcrB and ZipA are strikingly similar to those from analytical ultracentrifugation (AUC) of the same samples. Therefore SMA-PAGE has the potential to provide similar information to AUC but with modest amounts of protein and a simpler experimental set-up.

For some applications, the ability of SMA-PAGE to resolve aggregates and different oligomeric states gives it potential as a preparative method in its own right, rendering SEC wasteful and time-consuming by contrast. For both preparative and analytical applications, the SMA-PAGE

method has the potential to be faster and less demanding of sample quantity than other methods.

Native gel electrophoresis uses laboratory equipment that is inexpensive and routinely available. Therefore it provides a rapid and cost-effective means to identify the oligomeric state of membrane proteins and the constituents of membrane protein complexes. The compatibility of SMA-PAGE with immunoblotting is particularly valuable in the case of solubilised membranes, because SMA solubilisation is a minimally disruptive method (isolating small discs of bilayer-containing membrane proteins). This allows the size and composition of native assemblies of membrane proteins to be rapidly and confidently defined.

Finally, purified protein-SMALPs can be directly transferred from native gels onto grids for EM, a method previously described for soluble proteins by Knispel *et al* (2012)²⁹. Protein-SMALPs can also be extracted into buffer and then placed on grids for EM analysis. By reducing the demand for sample, and removing aggregated material, the SMA-PAGE method provides an opportunity to increase the efficiency of the protein production pipeline. Since cryo-EM now has the potential to generate structural data at a high resolution, including with SMALPs^{30–33}, this application could be transformative to the structural biology of membrane proteins. However, extensive optimisation of the SMA-PAGE method will be required to achieve this.

Conclusions

SMA-PAGE can be quickly carried out using equipment that is commonplace in life sciences laboratories, providing information that typically relies on time-consuming processes or expensive instruments. It is compatible with many important downstream analyses and applications in protein biochemistry. It applies specifically to membrane proteins, a class of proteins that is frequently challenging to overexpress and isolate, demanding very little sample and offering the answers to many pressing questions in the field. The method ties seamlessly into those already developed for solubilisation, purification and characterisation of membrane proteins using SMA, which have delivered success in recent years for many important targets.

Materials and Methods

1. Materials

Unless otherwise stated, chemicals were purchased from Sigma Aldrich or ThermoFisher. Styrene maleic anhydride 2:1 polymer (SMA2000P) was purchased from Grolman, U.K. and hydrolysed to styrene maleic acid as previously described ²⁸. Gels and gel tanks were purchased from BioRad (U.K). Handcast gels were made using 40% bisacrylamide (National Diagnostics). For native gels the NativeMark protein ladder between 20 kDa and 1280 kDa was used (ThermoFisher, U.K.). For SDS-PAGE we used NuPAGE LDS sample buffer (Invitrogen), HyperPage 10 -190 kDa molecular weight markers (Bioline, UK) and GE Healthcare high molecular weight markers (10-250 kDa). Grids for EM (Formvar/Carbon film on Copper 200 mesh) were purchased from EM Resolutions Ltd, U.K.

2. Purification and characterisation of proteins

Crude *E. coli* membranes were isolated according to standard protocols and solubilised in SMA as recently described ²⁸. Proteins purified in this study were all His-tagged and were isolated by nickel affinity chromatography. Protein-SMALPs were further fractionated and analysed by size-exclusion chromatography on the Superdex200 10/300 column in a buffer of 20 mM Tris-Cl pH 8, 150 NaCl. Protein-SMALPs were analysed by AUC to estimate their molecular weight/oligomeric state as previously described ²⁸.

3. Preparation of native acrylamide gels and native load dye

Several gel types were used for these experiments: native PAGE used 4-20 % precast gradient gels (Mini-Protean TGX and Criterion TGX, BioRad, U.K.) and hand cast gels at 10 % acrylamide, which did not use a stacking gel. All native gels were run using Tris/glycine running buffer (25 mM Tris pH 8.8, 192 mM glycine). The native loading dye was 1 mg/ml bromophenol blue, 20 mM Tris pH 8 and 50 % (v/v) glycerol. Native gels were run at 4 °C, 150 V for 60 minutes or until the dye front reached the bottom of the gel. SDS-PAGE experiments used 10 % gels and the running buffer previously described supplemented with 0.1 % (w/v) SDS. The sample buffer was 4x LDS (Invitrogen, U.K.) and gels were run at 180 V for 45 minutes at room temperature. To detect protein, gels were stained with InstantBlue (Expedeon, U.K.), silver stain or Ponceau S stain according to standard protocols and manufacturers' instructions.

4. Immunoblotting

Polyacrylamide gels run under native conditions were soaked for 10 minutes in SDS-PAGE running buffer prior to transfer. Proteins were transferred from the gel onto a nitrocellulose membrane using the iBlot system (BioRad, U.K.) at 20 V for 7 minutes. Membranes were blocked with a buffer containing 5 % (w/v) skimmed milk powder in phosphate-buffered saline (ThermoFisher, U.K.) supplemented with 0.1 % Tween20 (PBS-T). His-tagged proteins were probed using a 1:2000 dilution of the anti-6His antibody AP-conjugated (R932-25, ThermoFisher, U.K.) in blocking buffer and detected by enhanced chemiluminescence reagents (Pierce, UK) and a digital imaging system.

5. Electron microscopy

Grids for negative stain EM were prepared by transfer of protein directly from the SMA-PAGE gels, as an adaptation to the protocol of Knispel et al ²⁹. Briefly, samples were run in duplicate on one gel. Half was stained to determine the location of the protein band, and on the unstained gel, the protein-containing areas were cross hatched with a clean scalpel and wetted with 5 μ l buffer (20 mM Tris, 150 mM NaCl). EM grids were glow-discharged for 1 min, before being placed face down onto the unstained, wetted gels for 2 minutes. Grids were stained with 1 % uranyl acetate and micrographs were recorded at 50,000x magnification on a 200 kV JEOL 2011 transmission electron microscope. The cross-hatched areas on the gels were excised and submitted for mass spectrometry to confirm the identity of the proteins (see Section 6). Alternatively, bands were excised from gels, diced and incubated overnight at 4°C in 100 μ l buffer (20 mM Tris, pH 8, 150 mM NaCl). Gel fragments were removed by centrifugation (10,000 g, 10 min). The supernatant was used to prepare negatively stained grids according to standard protocols.

6. Excision of gel bands for mass spectrometry of lipid and protein

Bands containing the proteins of interest were excised from native PAGE gels using a clean scalpel. Three methods were used to extract protein from these gel bands. To analyse the samples recovered from native gels by SDS-PAGE, bands were diced with a scalpel and incubated with LDS loading dye and reducing agent before being run on standard SDS-PAGE gels to confirm their monomer molecular weights or subunit composition. Alternatively bands were homogenised in 5 % (w/v) SDS, and lipids were subsequently extracted and analysed by

LC-MSMS as described below. Finally, in some cases, gel bands were incubated with SEC buffer at 4 °C overnight and protein-SMALPs diffused into the buffer.

Lipid extraction

The lipid extraction was carried out based on a modified Folch method³⁴. Firstly, 200 µL ice-cold MeOH (supplemented with 0.005% w/v BHT) was added to 400 µL of sample, followed by 400 µL ice-cold CHCl₃ and lastly 150 µL ice-cold ultrapure H₂O. Each sample was vortexed for 20 s and sonicated in ice bath for 15 min after each solvent addition, to facilitate rigorous mixing and extraction. The samples were centrifuged at 16,000 x g for 5 min at 4 °C to achieve phase separation. The lipid-containing (lower) organic layer was collected and the remaining aqueous phase was subjected to a second extraction by adding 400 µL ice-cold CHCl₃. The samples were centrifuged again at 16,000 x g for 5 min at 4 °C and the organic layer was collected and combined with the first extraction. The resulting lipid extract was dried under a gentle stream of gas and reconstituted in 9:1 v/v MeOH:CHCl₃ for LC-MS/MS analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify lipids

The reconstituted lipid extract (10 µL) was injected onto a Luna® C8(2) 100 Å column, 150 x 1 mm (length x I.D.), 3 µm (particle size) (Phenomenex, USA) at a flow rate of 50 µL/min. Lipid separation was achieved using a gradient of the following mobile phases: THF:MeOH: 10 mM aq. ammonium acetate; A: 3:2:5 v/v/v; B: 7:2:1 v/v/v. The gradient was formulated as follows: 40% B for 4 min, 40% B to 60% B over 6 min, 60% B to 100% B over 15 min, and hold 100% B for 5 min, 100% B to 40% B over 2 min, then re-equilibrate to the 40% B over 13 min. The eluent was analysed on a QTRAP® 5500 mass spectrometer (SCIEX, Warrington, UK) operating in negative ion mode with instrument parameters as follows: curtain gas: 35 psi, ion spray voltage: -4.5 kV; temperature: 150°C; GS1: 13 psi; GS2: 0 psi; declustering potential: 50 V; entrance potential: 10 V; collision energy: 10 eV. The data were analysed using PeakView™ software (SCIEX).

Trypsin digestion and protein identification

In-gel digestion: After gel bands were excised, they were destained and digested by a robot system. Briefly, gel was destained with acetonitrile followed by 100 mM ammonium bicarbonate. This cycle was repeated if necessary until gel pieces were fully destained. Destained gel pieces were dried (vacuum centrifugation; 5 min) and rehydrated in 100 ammonium bicarbonate. Then

the gel pieces were reduced with 10 mM DTT, at 60°C for 15 min, the liquid was removed and replaced with 50mM iodoacetamide and 100 ammonium bicarbonate for alkylation. Gel pieces were incubated at room temperature in the dark for 45 min, washed with 100 ammonium bicarbonate and then dried for 5 min. Trypsin gold (Promega, WI, USA) solution was added in 100:1 ratio, shaken at room temperature for 20 min, before diluted with 100 mM ammonium bicarbonate. Hydrolysis was allowed to occur overnight (~16 h) at 37 °C. The gel pieces were first extracted with the solution of 2% acetonitrile and 0.1% formic acid in water and shaken for 30 minutes at room temperature. Extract the remaining peptides in the gel by using 70% acetonitrile with 0.1% formic acid in water shaken for 30 minutes at room temperature. The supernatant was pooled and dried in an evaporator. The samples were re-suspended in 0.1% formic acid in water for the LC-MS/MS analysis.

In-solution digestion: Protein samples of 10 µg were dissolved in 100mM ammonium bicarbonate (pH 8). 25 µL of 10 mM dithiothreitol (DTT) was added and the sample was incubated at 60 °C for 45 mins. Sample was cooled to room temperature and cysteines alkylated by addition of 25µl 50mM iodoacetamide, mixed and incubated at room temperature in the dark for 45 mins. 50 µl of trypsin gold (Promega, Southampton, Hampshire, UK, 5 ng/µl) was subsequently added to the sample and incubated at 37 °C overnight. The digested sample was dried and resuspended in 0.1% formic acid, then desalted using C18 ZipTips . The ZipTip was prepared by pre-wetting in 2x 10 µL 100% acetonitrile and rinsing in 2x10 µL 0.1% formic acid. Sample was loaded into the tip by aspirating and dispensing the sample 7-10 times. Next the tip was washed twice with 10µl 0.1% formic acid to remove excess salts before elution of peptides with 10µL of 50% acetonitrile/water/0.1% formic acid 3 times. Samples were dried down, then re-suspended in 10µL of 0.1% formic acid solution in water for LC-MS/MS analysis.

Protein identification by LC-MS/MS: UltiMate® 3000 HPLC series (Dionex, Sunnyvale, CA USA) was used for peptide concentration and separation. Samples were trapped on Thermo C18 PepMap 100 column, 3 µm particle size, 75µm i.d. x 2 cm and then separated on Thermo Nano Series™ standard columns (75 µm i.d. x 15 cm packed with C18 PepMap100, 3 µm, 100Å). The gradient for elution was 3.2% to 44% solvent B (0.1% formic acid in acetonitrile) for 30 min. Peptides were eluted directly (350 nL/min) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into a LTQ Orbitrap Elite mass spectrometer (ThermoFisher Scientific). The mass spectrometer alternated between a full FT-MS scan (m/z 380 – 1800) and subsequent collision-induced dissociation (CID) MS/MS scans of the 7 most abundant ions. The MS and MS/MS scans were searched against the Uniprot database using Proteome Discoverer

1.4 (ThermoFisher Scientific) with SequestHT algorithm. The precursor mass tolerance was 10 ppm and the MS/MS mass tolerance was 0.8 Da. Two missed cleavages were allowed and the data were filtered with a strict false discovery rate (FDR) of 0.01 and a relaxed FDR of 0.05. The protein grouping filter was applied and a minimum of two high confidence peptides (strict FDR 0.01) were accepted as a real hit.

Acknowledgments

A.C.K.T. acknowledges funding from the European Union under a Marie Skłodowska-Curie Initial Training Network FP7-PEOPLE-2012-ITN Grant Agreement Number 316630 CAS IDP. N.P. was funded by the BBSRC Tools and Resources Development Fund grant number BB/P027482/1. We thank the proteomics facility at the University of Birmingham for assistance with this project.

Author contributions

N.L.P designed the study and carried out all experiments except mass spectrometry, which was undertaken by C.M.S and A.C.K.T., and electron microscopy which was carried out by K.S., R.C., S.E.B. and S.P.M. M.R., M.P., P.S., S.C.L., T.J.K and J.M.E. and V.P. contributed protein samples. N.L.P and T.R.D wrote the manuscript. C.H.B, G.H., D.I.R., C.J.I.S, T.J.K. C.M.S., J.M.E and V.P. contributed to editing the manuscript.

Competing interests

We declare no competing interests.

References

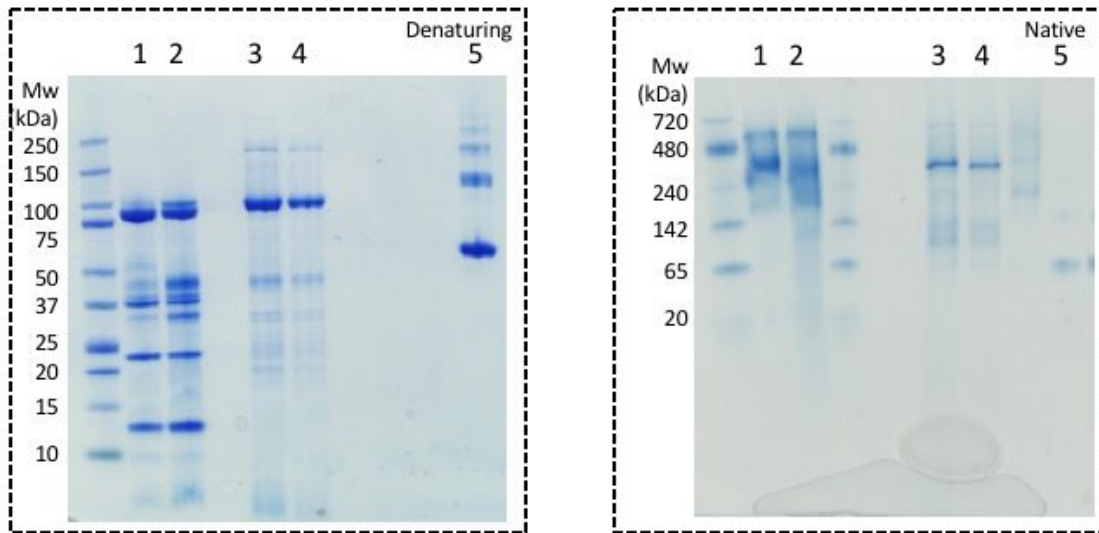
1. Suzuki, N. *et al.* An efficient screening method for purifying and crystallizing membrane proteins using modified clear-native PAGE. *Anal. Biochem.* **548**, 7–14 (2018).
2. Wittig, I. & Schägger, H. Advantages and limitations of clear-native PAGE. *Proteomics* **5**, 4338–4346 (2005).
3. Eubel, H. & Millar, A. H. Systematic Monitoring of Protein Complex Composition and Abundance by Blue-Native PAGE. *Cold Spring Harb. Protoc.* **2009**, db.prot5221–pdb.prot5221 (2009).
4. Faull, S. V., Brown, A. E., Haq, I. & Irving, J. A. Electrophoresis- and FRET-Based Measures of Serpin Polymerization. *Methods Mol. Biol.* **1639**, 235–248 (2017).
5. Dafforn, T. R., Mahadeva, R., Elliott, P. R., Sivasothy, P. & Lomas, D. A. A kinetic mechanism for the polymerization of α 1-antitrypsin. *J. Biol. Chem.* **274**, 9548–9555 (1999).
6. Crichton, P. G., Harding, M., Ruprecht, J. J., Lee, Y. & Kunji, E. R. S. Lipid, detergent, and Coomassie Blue G-250 affect the migration of small membrane proteins in blue native gels: mitochondrial carriers migrate as monomers not dimers. *J. Biol. Chem.* **288**, 22163–22173 (2013).
7. Wittig, I., Karas, M. & Schägger, H. High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. *Mol. Cell. Proteomics* **6**, 1215–1225 (2007).
8. Wittig, I., Braun, H.-P. & Schägger, H. Blue native PAGE. *Nat. Protoc.* **1**, 418–428 (2006).
9. Knowles, T. J. *et al.* Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. *J. Am. Chem. Soc.* **131**, 7484–7485 (2009).
10. Smirnova, I. A. *et al.* Isolation of yeast complex IV in native lipid nanodiscs. *Biochim. Biophys. Acta* **1858**, 2984–2992 (2016).

11. Dörr, J. M. *et al.* Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K⁺ channel: the power of native nanodiscs. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 18607–18612 (2014).
12. Gulati, S. *et al.* Detergent free purification of ABC transporters. *Biochem. J* **44**, 1–24 (2014).
13. Swainsbury, D. J. K., Scheidelaar, S., van Grondelle, R., Killian, J. A. & Jones, M. R. Bacterial reaction centers purified with styrene maleic acid copolymer retain native membrane functional properties and display enhanced stability. *Angew. Chem. Int. Ed Engl.* **53**, 11803–11807 (2014).
14. Long, A. R. *et al.* A detergent-free strategy for the reconstitution of active enzyme complexes from native biological membranes into nanoscale discs. *BMC Biotechnol.* **13**, 41 (2013).
15. Jamshad, M. *et al.* G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. *Biosci. Rep.* **35**, (2015).
16. Postis, V. *et al.* The use of SMALPs as a novel membrane protein scaffold for structure study by negative stain electron microscopy. *Biochim. Biophys. Acta* **1848**, 496–501 (2015).
17. Komar, J. *et al.* Membrane protein insertion and assembly by the bacterial holo-translocon SecYEG-SecDF-YajC-YidC. *Biochem. J* **473**, 3341–3354 (2016).
18. Prabudiansyah, I., Kusters, I., Caforio, A. & Driessen, A. J. M. Characterization of the annular lipid shell of the Sec translocon. *Biochim. Biophys. Acta* **1848**, 2050–2056 (2015).
19. Martos, A. *et al.* Characterization of self-association and heteroassociation of bacterial cell division proteins FtsZ and ZipA in solution by composition gradient-static light scattering. *Biochemistry* **49**, 10780–10787 (2010).
20. Dawson, R. J. P. & Locher, K. P. Structure of the multidrug ABC transporter Sav1866 from *S. aureus* in complex with AMP-PNP. (2007). doi:10.2210/pdb2onj/pdb

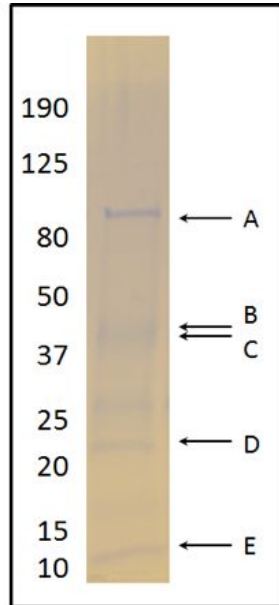
21. Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. Crystal structure of Bacterial Multidrug Efflux transporter AcrB. (2002). doi:10.2210/pdb1iwg/pdb
22. Iadanza, M. G. *et al.* E. coli BAM complex (BamABCDE) by cryoEM. (2016). doi:10.2210/pdb5ljo/pdb
23. Anwari, K. *et al.* A modular BAM complex in the outer membrane of the alpha-proteobacterium *Caulobacter crescentus*. *PLoS One* **5**, e8619 (2010).
24. RayChaudhuri, D. ZipA is a MAP–Tau homolog and is essential for structural integrity of the cytokinetic FtsZ ring during bacterial cell division. *EMBO J.* **18**, 2372–2383 (1999).
25. Gulati, S. *et al.* Detergent-free purification of ABC (ATP-binding-cassette) transporters. *Biochem. J* **461**, 269–278 (2014).
26. Parmar, M. *et al.* Using a SMALP platform to determine a sub-nm single particle cryo-EM membrane protein structure. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1860**, 378–383 (2018).
27. Sun, C. *et al.* Structure of the alternative complex III in a supercomplex with cytochrome oxidase. *Nature* **557**, 123–126 (2018).
28. Lee, S. C. *et al.* A method for detergent-free isolation of membrane proteins in their local lipid environment. *Nat. Protoc.* **11**, 1149–1162 (2016).
29. Knispel, R. W., Kofler, C., Boicu, M., Baumeister, W. & Nickell, S. Blotting protein complexes from native gels to electron microscopy grids. *Nat. Methods* **9**, 182–184 (2012).
30. Parmar, M. *et al.* Using a SMALP platform to determine a sub-nm single particle cryo-EM membrane protein structure. *Biochim. Biophys. Acta* **1860**, 378–383 (2018).
31. Efremov, R. G., Gatsogiannis, C. & Raunser, S. Lipid Nanodiscs as a Tool for High-Resolution Structure Determination of Membrane Proteins by Single-Particle Cryo-EM. *Methods Enzymol.* **594**, 1–30 (2017).

32. Kühlbrandt, W. Biochemistry. The resolution revolution. *Science* **343**, 1443–1444 (2014).
33. Sun, C. *et al.* cryo-EM Structure of Alternative Complex III/ AA 3 Cytochrome Oxidase Supercomplex from *Flavobacterium Johnsoniae*. *Biophys. J.* **114**, 43a (2018).
34. Reis, A. *et al.* A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. *J. Lipid Res.* **54**, 1812–1824 (2013).

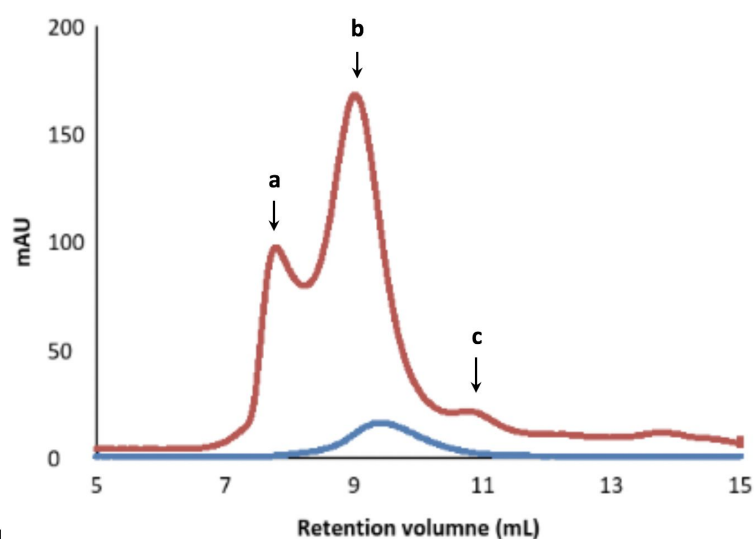
Supplementary Figures and Tables



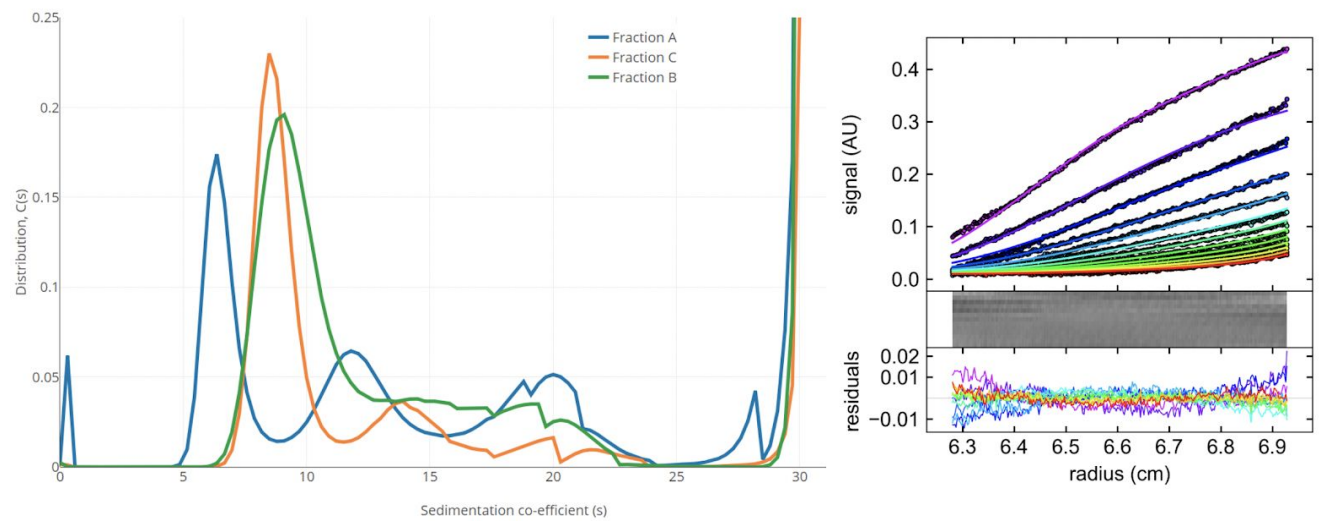
Supplementary Figure 1: Comparison of native and denaturing gels loaded with the Bam complex, AcrB and BSA showing the full gel and molecular weight markers. Each gel was loaded as follows: 1) Bam complex, 2) Bam complex, 3) AcrB 2ug 4) AcrB 1 ug, 5) BSA 1 ug



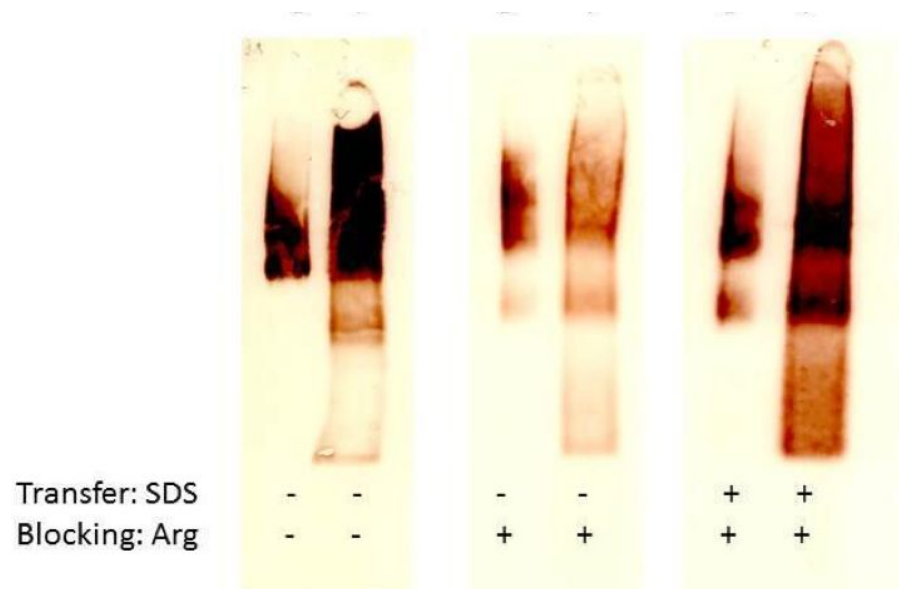
Supplementary Figure 2: Evidence for soluble subunits co-migrating on native PAGE with membrane proteins-SMALPs. The Bam complex was excised from a native gel, extracted into denaturing load dye and the subunits were resolved by SDS-PAGE. BamA-SMALPs were recovered along with the soluble subunits (BamB-E) as indicated by the letters on the right hand side.



Supplementary Figure 3: Size exclusion chromatography of pure Sav1866-SMALPs. Pure Sav1866-SMALPs were fractionated on a Superdex200 column and protein was detected using its absorbance at 280 nm (red trace). Fractions from the peaks labelled a, b and c were analysed further using SDS-PAGE, SMA-PAGE and svAUC. A sample from peak b was reinjected onto the column and resolved using the same buffer (blue trace).



Supplementary Figure 4: Sedimentation velocity analytical ultracentrifugation of Sav1866. Three fractions (a,b,c) of purified Sav1866-SMALPs were analysed by svAUC to identify differences between protein eluting from an SEC column at different retention volumes (see Supp. Fig. 1). The sedimentation of the sample was monitored using interference, and the data were analysed using Sedfit (REF) to determine the distribution of sizes of the protein in each sample (A). An example of the data fitting for Fraction A is also shown (B).



Supplementary Figure 5: Optimised immunoblotting of ZipA samples separated by SMA-PAGE. In each case “S” indicates crude solubilised membranes and “P” indicates pure protein. Incubation of the gel with sodium dodecylsulphate prior to transfer is indicated below the blot (SDS +/-). Inclusion of 25 mM arginine in the blocking buffer is indicated (Arg +/-).

Supplementary Table 1: Proteins used to assess the applicability of the SMA-PAGE method

Protein	Monomer Mw (kDa)	Functional state/minimal functional unit oligomeric	Theoretical isoelectric point (pI)
ZipA	36	Monomers and higher oligomers ¹⁹	5.6
Sav1866	65	Homodimer ²⁰	6.7
AcrB	112	Homotrimer ²¹	5.4
Bam A/B/C/D/E	91/42/37/28/12 (211)	Heteropentamer ²²	4.9

Supplementary Table 2: Identification of components of the Bam complex in SMA-PAGE extracts. A band from an SMA-PAGE gel containing the Bam complex was excised and underwent in-gel trypsin digestion followed by detection by LC-MSMS. The table shows the top matches in the samples cut off at a score of 20. Components of the Bam complex (Bam A-E) are highlighted in red.

Description	Molecular weight [kDa]	Coverage (% of protein sequence)	Unique peptides detected	Score
Outer membrane protein assembly factor BamA	90.5	57.53	42	279.06
Outer membrane protein assembly factor BamC	36.8	83.14	17	144.75
Multidrug efflux pump subunit AcrB	113.5	31.36	24	105.04
Chaperone SurA	47.3	37.38	12	89.40
Outer membrane protein assembly factor BamB	41.9	44.39	13	70.87
Outer membrane protein assembly factor BamD	27.8	61.22	13	55.82
NADH-quinone oxidoreductase subunit C/D	68.2	26.51	13	39.47
Cytochrome bo(3) ubiquinol oxidase subunit 1	74.3	14.33	9	35.80
Cytochrome bo(3) ubiquinol oxidase subunit 2	34.9	17.46	3	35.02
Maltodextrin phosphorylase	90.5	16.06	12	30.73
Outer membrane protein assembly factor BamE	12.3	17.70	1	22.72
Catalase HP11 OS=Escherichia coli (strain K12)	84.1	13.81	9	20.57

Supplementary Table 3: Identification of AcrB transferred to electron microscopy grids.

After protein was transferred from an area of the gel onto an EM grid, the same area of the gel was excised for protein identification by mass spectrometry. A total of 68 proteins were identified and those with a score of twenty or above are displayed in the table. The target protein AcrB is highlighted in red.

Description	Molecular weight [kDa]	Coverage (% of protein sequence)	Unique peptides detected	Score
Multidrug efflux pump subunit AcrB	113.5	42.90	34	382.89
D-methionine-binding lipoprotein MetQ	29.4	36.53	6	31.76
2-oxoglutarate dehydrogenase E1 component	105.0	10.18	9	30.37
Cytochrome bo(3) ubiquinol oxidase subunit 2	34.9	17.46	3	23.94
Cytochrome bo(3) ubiquinol oxidase subunit 1	74.3	8.30	5	20.12